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# Development of a consensus method for culture of *Clostridium difficile* from meat and its use in a survey of U.S. retail meats

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## Abstract

Three previously described methods for culture of *Clostridium difficile* from meats were evaluated by microbiologists with experience in *C. difficile* culture and identification. A consensus protocol using BHI broth enrichment followed by ethanol shock and plating to selective and non-selective media was selected for use, and all participating laboratories received hands-on training in the use of this method prior to study initiation. Retail meat products (N = 1755) were cultured for *C. difficile* over 12 months during 2010-2011 at 9 U.S. FoodNet sites. No *C. difficile* was recovered, although other clostridia were isolated.

### Keywords

*Clostridium difficile*; Retail meat; Contamination

## 1. Introduction

*Clostridium difficile* is an important cause of infectious diarrhea in healthcare settings, usually following antimicrobial therapy. However, *C. difficile* infection (CDI) is an increasingly-recognized cause of diarrhea among people in community settings without recent inpatient hospital exposure (Centers for Disease Control and Prevention, 2008; Kutty et al., 2010; Lambert et al., 2009). Hyper-virulent *C. difficile* strains have been associated

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with increased incidence and severity of CDI in healthcare settings (Deneve et al., 2009; McDonald et al., 2005); increases in community-associated CDI (CA-CDI) may be driven by other factors. *C. difficile* causes disease in food animals (Debast et al., 2009; Keel et al., 2007; Songer, 2004) and has been recovered from retail foods in several countries (de Boer et al., 2011; Harvey et al., 2011a, 2011b; Johnson et al., 2005; Metcalf et al., 2010; Rodriguez-Palacios et al., 2007; Songer et al., 2009), leading some to suggest that food may be a source for CA-CDI (Metcalf et al., 2010; Rupnik, 2007; Rupnik and Songer, 2010). Although several groups have reported the isolation of *C. difficile* from retail meats (Harvey et al., 2011a, 2011b; Rodriguez-Palacios et al., 2009; Rodriguez-Palacios et al., 2007; Songer et al., 2009; Weese et al., 2009), there is no consensus method for culture of *C. difficile* from meats or other food products. This study was designed to establish a consensus method for culture of *C. difficile* from meats, and to determine the prevalence of *C. difficile* contamination of selected retail meats in the United States using a standardized culture method.

## 2. Materials and methods

### 2.1. Comparison of culture methods for *C. difficile* culture from meats

Several different culture methods were evaluated for recovery of *C. difficile* from spiked ground meat samples. Independently-acquired ground beef was inoculated using a single spore suspension at a rate of approximately 100 spores/gram in three laboratories with expertise culturing and characterizing C. difficile. The spore suspension was prepared as described previously (Bertolo et al., 2012), except that phase contrast microscopy was not performed. Three types of initial broth enrichment were tested: 1) *C. difficile* Moxalactam Norfloxacin (CDMN) broth (ThermoFisher Scientific, Waltham, MA) + 0.1% Taurocholate (SigmaeAldrich, St. Louis, MO) without heat shock; 2) Brain Hearth Infusion (BHI) broth (ThermoFisher Scientific) + 0.1% Taurocholate (BHIT) without heat shock; and 3) BHIT including a heat shock step. Ten grams of ground beef were inoculated into 90 mL enrichment broth in sterile cups, tightly capped and mixed by inversion. Initial heat shock was performed by immersing the vessel in an 80 °C water bath for 30 min. Enrichment cultures were incubated anaerobically at 35 °C.

Each broth enrichment culture was sampled on days 1, 3, and 5 by direct plating, and by plating following ethanol shock or heat shock. For ethanol shock, 1 mL of the liquid enrichment culture was added to 1 mL 95% ethanol, and mixed at room temperature every 15 min for 1 h. For heat shock, 1 mL enrichment culture was placed in a sterile tube and incubated 10 min at 80 °C. One mL of each enrichment broth or shocked culture sample was centrifuged at 3800 × *g* for 10 min, supernatant fluid was decanted and the pellet was resuspended in the remaining liquid. One drop of this sediment was inoculated onto anaerobic Blood Agar (anaBAP) (PathCon Laboratories, Norcross, GA), Cycloserine Cefoxitin Fructose Agar with Taurocholate (CCFA-ST; ThermoFisher), and CDMN agar with Taurocholate. Plates were incubated anaerobically at 35 °C and examined at 48 and 96 h for growth of *C. difficile* colonies.

In order to assess the impact of taurocholate in the enrichment step, two laboratories evaluated BHI broth with and without taurocholate using the three aliquots of spiked meat

samples described above for BHIT without heat shock, except that enrichment broths were cultured only after 3 and 5 days incubation.

### 2.2. Retail meats sampling

The Foodborne Diseases Active Surveillance Network (FoodNet) conducts ongoing surveillance for foodborne pathogens in retail foods through a network of participating sites across the United States. Meats were purchased each month for 12 consecutive months at retail establishments in eight FoodNet states (CA, CO, CT, MD, MN, NY, OR, TN) and Pennsylvania, from 2009 through 2011. Each site obtained 5 to 10 samples of fresh, non-frozen ground beef and ground turkey each month from a random sample of five grocery stores within the local catchment area. At some sites, pork chops and chicken breasts were also sampled at some of the time points. The store name, product lot number (if available), sell-by date, purchase date, and laboratory processing date were recorded for each meat sample that was processed for culture. Samples were kept cold in a cooler with ice packs during transport from the grocery store to the laboratory for testing.

### 2.3. Culture of retail meats for C. difficile

Prior to the initiation of *C. difficile* surveillance activities, laboratory personnel from each of the participating FoodNet sites received two days of intensive, hands-on laboratory training with the standardized method to ensure consistency.

A total of 1755 retail meat samples were cultured for *C. difficile.* Ten grams of ground meat (turkey/beef) were suspended in 90 mL of BHI broth and inverted to mix. Intact meats (pork chops/chicken breasts) were rinsed in 225 mL Buffered Peptone Water, and 50 mL of the rinse was added to 50 mL of double-strength BHI broth, then incubated at 35e37 °C for 3e7 days under anaerobic conditions. After incubation, the broth was ethanol shocked as described in Section 2.1, inoculated to anaBAP and CCFA-ST, and incubated anaerobically. Another set of anaBAP and CCFA-ST plates was inoculated with a drop from the original meat/enrichment broth without ethanol shock. All plates were examined at 48-72 h for colonies characteristic of C. difficile: cream-colored on anaBAP or yellow (fructose fermenting) on CCFA, irregular, non-hemolytic, ground glass colonies that fluoresce yellow-green under UV light with a *p*-cresol odor. Plates without characteristic colonies were re-incubated for a maximum of 96 h total. Colonies suggestive of *C. difficile* were gram stained and subcultured aerobically and anaerobically, and potential *C. difficile* isolates were shipped to the Centers for Disease Control and Prevention (CDC) Anaerobic Reference Laboratory for confirmation as *C. difficile* (yellow colonies on CCFA, indole negative, L-proline aminopeptidase positive, with the characteristic *p*-cresol odor) and characterization by pulsed-field gel electrophoresis and toxin PCR. To ensure the validity of the culturing and sampling methods, the CDC Anaerobe Reference Laboratory used the standardized method to culture ground beef and ground turkey samples purchased by the Georgia FoodNet site during January through March, 2011, for a total of 60 samples tested.

## 3. Results and discussion

### 3.1. Selection of a consensus method for culture of *C. difficile* from meats

*C. difficile* was recovered from the spiked meat samples with each of the methods evaluated. The best recovery was observed after 3 and 5 days of enrichment in broth medium (Table 1). Recovery of *C. difficile* in BHIT broth without initial heat shock appeared to be better than in CDMN broth or in BHIT with initial heat shock (Table 1). A subsequent evaluation of BHI compared with BHIT demonstrated no added benefit of taurocholate on *C. difficile* recovery in the broth enrichment step (data not shown). There was no noticeable difference in the performance of the three plating protocols or agar media that we evaluated. Based on equivalent performance, media costs, and ease of use, a standardized method was selected for use in the larger study. This method involved 3-5 days of enrichment in BHI broth, after which an aliquot was subjected to alcohol shock, and both the shocked and untreated aliquots were plated to anaBAP and CCFA-ST agars.

### 3.2. Results of culture at FoodNet sites and CDC

A total of 1755 retail meats were sampled over 12 months (Table 2), including 617 ground beef, 614 ground turkey, 259 chicken breasts, and 265 pork chops. Forty-four potential *C. difficile* isolates were sent to CDC from FoodNet sites, but of these, none was confirmed as *C. difficile*. As a supplemental investigation, the Anaerobe Reference Laboratory at CDC performed three months of culture on ground beef and ground turkey samples purchased by the Georgia FoodNet site using consensus culturing methods. From the 60 samples that were tested, no *C. difficile* were recovered at CDC, although other clostridia were isolated. During one month of sampling, clostridia were recovered from 16 of 20 (80%) ground meat samples, six of which yielded multiple species, including *Clostridium sporogenes, Clostridium cadaveris, Clostridium perfringens, Clostridium bifermentans, Clostridium septicum*, and two unidentified Clostridium species (Table 3). All 10 ground turkey samples cultured were positive for *C. sporogenes*, four of which also contained another Clostridium species. Six of the ground beef cultures were positive for Clostridium species, two of which contained more than one species.

### 3.3. Limitations

This study has several limitations. Only three replicates from a single *C. difficile* spore suspension and negative controls were evaluated by three experienced laboratories participating in the method development study. Thus, the method was evaluated for only one strain type. Although our comparison demonstrated no impact of sodium taurocholate in the broth enrichment medium, it is possible that some *C. difficile* strains may have benefited from its presence. Because the methods used in this study included broth enrichment as a first step in culture, no attempt was made to quantitate the number of bacteria. It is possible that the enrichment broths evaluated might have had subtle effects on spore germination that could have been appreciated with a quantitative culture method. Finally, many of the laboratories participating in the large surveillance study had little or no prior experience in culture of *C. difficile*. To address this, hands-on training in *C. difficile* culture was conducted before the study began and each was provided with a positive control strain of *C. difficile*.

As a further control, three months of surveillance culture was performed at CDC on meats collected through the GA FoodNet program, and no *C. difficile* was isolated.

### 3.4. Conclusions

In conclusion, comprehensive surveying of retail meats from across the United States suggest that *C. difficile* is not a common contaminant of retail meat products in the United States, although other Clostridium species were commonly found. Our study provides a standardized method for culture of *C. difficile* and other Clostridium species from retail meats, which we hope will serve to help remove the confounding effects of different culture protocols on *C. difficile* recovery rates. Our findings differ from those reported in other studies of *C. difficile* in U.S. meat products, and may in part reflect regional differences since those studies were conducted in limited geographic settings (Harvey et al., 2011a, 2011b; Songer et al., 2009). Nonetheless, these data indicate a low prevalence of *C. difficile* among U.S. retail meat products.

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## Table 1

**Number of inoculated meat samples[a] positive for *C. difficile* with each method evaluated**

| Shock method | Plate medium | 1 day enrichment | 3 day enrichment | 5 day enrichment |
|---|---|---|---|---|
| **Enrichment in CDMN + 0.1% Taurocholate** | | | | |
| Ethanol | AnaBAP | 1/3 | 3/3 | 3/3 |
| Ethanol | CCFA-ST | 0/3 | 2/3 | 3/3 |
| Ethanol | TCDMN | 0/3 | 3/3 | 3/3 |
| Heat | AnaBAP | 0/3 | 1/3 | 3/3 |
| Heat | CCFA-ST | 0/3 | 1/3 | 1/3 |
| Heat | TCDMN | 0/3 | 1/3 | 3/3 |
| None | CCFA-ST | 0/3 | 3/3 | 2/3[b] |
| None | TCDMN | 0/3 | 3/3 | 3/3 |
| **Enrichment in BHIT + 0.1% Taurocholate, with initial heat shock** | | | | |
| Ethanol | AnaBAP | 2/3 | 2/3 | 2/3 |
| Ethanol | CCFA-ST | 2/3 | 2/3 | 2/3 |
| Ethanol | TCDMN | 3/3 | 2/3 | 2/3 |
| Heat | AnaBAP | 1/3 | 2/3 | 2/3 |
| Heat | CCFA-ST | 1/3 | 2/3 | 2/3 |
| Heat | TCDMN | 1/3 | 2/3 | 2/3 |
| None | CCFA-ST | 1/3 | 2/3 | 2/3[c] |
| None | TCDMN | 1/3 | 2/3 | 2/3[c] |
| **Enrichment in BHIT + 0.1% Taurocholate, without heat shock** | | | | |
| Ethanol | AnaBAP | 2/3 | 3/3 | 3/3 |
| Ethanol | CCFA-ST | 2/3 | 3/3 | 3/3 |
| Ethanol | TCDMN | 2/3 | 3/3 | 3/3 |
| Heat | AnaBAP | 3/3 | 3/3 | 3/3 |
| Heat | CCFA-ST | 3/3 | 3/3 | 3/3 |
| Heat | TCDMN | 3/3 | 3/3 | 3/3 |
| None | CCFA-ST | 3/3[b] | 3/3 | 3/3[b] |
| None | TCDMN | 3/3 | 3/3 | 3/3 |

AnaBAP: Anaerobe blood agar; CCFA-ST: Cycloserine cefoxitin fructose agar-sodium taurocholate; TCDMN: Taurocholate *C. difficile* moxalactam norfloxacin agar.

[a] Each inoculated sample was tested in an independent laboratory.

[b] *C. difficile* was recovered, but at least one culture was contaminated with other organisms.

[c] In one laboratory, no *C. difficile* recovered but contaminating organisms were recovered.

**Table 2**

**Results of culture for *C. difficile* among U.S. retail meat samples**

| Meat type | Tested by state public health laboratories | | | Tested by CDC | |
|---|---|---|---|---|---|
| | Total sampled | Potential *C. difficile*[a] | Confirmed *C. difficile* | Total sampled | *C. difficile* |
| Ground Beef | 617 | 31 | 0 | 30 | 0 |
| Ground Turkey | 614 | 14 | 0 | 30 | 0 |
| Chicken Breast | 259 | 1 | 0 | 0 | Not tested |
| Pork Chops | 265 | 9 | 0 | 0 | Not tested |
| Total | 1755 | 55 | 0 | 60 | 0 |

[a]Potential *C. difficile* isolates submitted to CDC for confirmation.

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**Table 3**

**Clostridium species isolated from ground meats cultured at CDC during March, 2011**

| | Ground beef (n = 10) | Ground turkey (n = 10) |
|---|---|---|
| *C. sporogenes* | 3 | 10 |
| *C. cadaveris* | 0 | 3 |
| *C. perfringens* | 2 | 0 |
| *C. bifermentans* | 0 | 1 |
| *C. septicum* | 1 | 0 |
| *C. difficile* | 0 | 0 |
| Other *Clostridium* spp. | 2 | 0 |
| Total Clostridium isolates | 8 | 14 |
| Total meat samples positive for Clostridium[a] | 6 | 10 |

[a]2 Ground beef and 4 ground turkey samples contained more than one *Clostridium* species.